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Kozielewicz, Pawel; Grafton, Gillian; Kutner, Andrzej; Curnow, John; Gordon, John; Barnes, Nicholas

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Author: Paweł Kozielewicz Gillian Grafton Andrzej Kutner S. John Curnow John Gordon Nicholas M. Barnes

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Novel vitamin D analogues; cytotoxic and anti-proliferative activity against a diffuse large B-cell lymphoma cell line and B-cells from healthy donors

Paweł Kozielewicz[a,,b,], Gillian Grafton[a,b], Andrzej Kutner[c], S. John Curnow[a,d], John Gordon[a] and Nicholas M. Barnes[a,b]*

[a] *Celentyx Ltd, Birmingham Research Park, Birmingham B15 2SQ UK*

[b] *School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, B15 2TT UK*

[c] *Pharmaceutical Research Institute, Warsaw 02-785, Poland*

[d] *School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, B15 2TT UK*

* Corresponding author: p.kozielewicz@pgr.bham.ac.uk

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Highlights

- We have examined the action of 1,25D3 and vitamin D analogues (VDAs) on malignant and healthy B cells.
- 1,25D_3 and VDAs displayed moderate cytotoxic and pro-apoptotic actions upon DLBCL cells.
- 1,25D_3 and VDAs displayed concentration and time-dependent anti-proliferative actions upon stimulated B-cells from healthy donors.
- VDAs may offer therapeutic potential for the treatment of DLBCL or conditions benefitted by B-cell depletion.

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Abstract

Calcitriol (1,25-dihydroxyvitamin D_3, 1,25D_3) and vitamin D side-chain modified analogs (VDAs) have gained considerable attention as potential drugs in the treatment of acute myeloid leukemia (AML), yet studies of the impact of 1,25D_3 and VDAs upon other haematological malignancies are more limited. To address this gap in knowledge, we have examined the action of 1,25D_3 and VDAs on a human cell line (DOHH2, K422) typifying diffuse large B-cell lymphoma (DLBCL) and also peripheral blood B-cells isolated from healthy donors.

1,25D_3 and certain VDAs displayed moderate cytotoxic and pro-apoptotic actions upon DLBCL cells. 1,25D_3 and VDAs (100 nM) caused the death of approximately 40% DOHH2 cells after 24 hours stimulation, similar to their impact on HL-60 cells (acute myeloid leukaemia cell line). In addition, 1,25D_3 and VDAs displayed concentration and time-dependent anti-proliferative actions upon stimulated B-cells from healthy donors. The VDAs inhibited proliferation by approximately 30%. Hence VDAs may offer therapeutic potential for the treatment of DLBCL or conditions benefitted by B-cell depletion.

Keywords: vitamin D analogues; B-cells; Diffuse Large B-cell lymphoma; proliferation; drug synergy

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1. Introduction

Calcitriol (1,25-dihydroxyvitamin D_3; 1,25D_3) impacts physiological processes in the human body, with perhaps the most notable being the regulation of Ca^{2+} and phosphate (P_i) transport and bone mineralization [1]. Other actions include control of the growth, differentiation and functional activity of numerous cell types including those of the skin, immune system and pancreas [2].

Numerous studies demonstrate that the active form of vitamin D promotes the differentiation of HL-60 cells (a cell line that typifies acute myeloid leukaemia AML) to a phenotype resembling differentiated monocytes in bone marrow [3], such that majority of cells are now CD14+ cells [4]. Similar results have been obtained for cell lines of biologically related origin, e.g. THP-1 and U-937 cells [5, 6]. In addition to the pro-differentiative effects of 1,25D_3, considerable attention has been paid to its cytotoxic and anti-proliferative actions. This has been studied in various mouse models, human cultured breast and colon cancers and also in HL-60 cells, which growth arrest in the G1 phase of cell cycle [7-9]. It should be noted however that the antitumor activity of 1,25D_3 is evident at supra-physiological concentrations, which also promote hypercalcemia and hypercalciuria [10], which are likely to limit therapeutic potential. This has motivated the search for vitamin D analogues (VDAs) with less propensity to increase Ca^{2+} levels. Some identified compounds retain interaction with the VDR and the anti-proliferative/pro-differentiative capacity but display more limited effect on calcium levels in comparison to 1,25D_3 [11].

Lymphoma is a cancer of lymphocytes presenting as either Hodgkin's (HL) or non-Hodgkin's lymphoma (NHL); over 90% of all NHL are of B-cell origin. According to the

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Cancer Research UK statistics over 12,000 people are diagnosed with NHL every year in the UK which makes it the 6th most common cancer in this country and the 11th most common cause of cancer death worldwide. As many as 40% of people diagnosed with this disease are predicted to survive less than 10 years.

It is estimated that 50% of NHL cases in the UK and 25-35% worldwide can be categorised as diffuse large B-cell lymphoma (DLBCL) [12]. DLBCL is an aggressive malignancy of B lymphocytes of germinal centre origin and is characterised by the presence of rapidly proliferating large cells with basophilic cytoplasm and vesicular nuclei [13]. Current treatments for DLBCL include the anti-CD20 monoclonal antibody, rituximab, and cycles of a combination of cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP). Unfortunately, rituximab-CHOP treatment (R-CHOP) can be associated with severe toxicity and only displays failure-free survival of 65% after 3 years [14].

There is little data concerning the therapeutic potential of vitamin D analogues for the treatment of NHL. To address further therapeutic potential from $1,25D_3$ and VDAs, we investigate in the present study the impact of $1,25D_3$ and novel VDAs upon DOHH2 and K422, cell lines representative of DLBCL, as well as B-cells from healthy donors.

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2. Experimental

2.1 Vitamin D analogues

1,25D$_3$ and all analogues (PRI-1890, PRI-1906, PRI-2191, PRI-2205; Fig. 1) were synthesized in the Pharmaceutical Research Institute (Warsaw, Poland). The compounds were dissolved in absolute ethanol, the concentration adjusted to 100 µM and the aliquots stored at -20ºC prior to experiments.

2.2 Cell culture

HL-60 and DOHH-2 cells were cultured in RPMI-1640 medium (Sigma Aldrich, UK) supplemented with 1 % L-glutamine (Life Technologies), 1 % penicillin/streptomycin (Sigma Aldrich, 10000 units penicillin and 10 mg streptomycin per ml) and 10 % heat-inactivated fetal bovine serum (later referred as: FBS HI, Sigma Aldrich). Cells were subcultured at a density of 1 – 1.5 x 10^6 cells/ml.

2.3 Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated from whole blood from healthy donors or from Leukocyte Reduced System (LRS) cones using Ficoll–Paque (GE Healthcare). 20 ml of blood was layered on 25 ml of Ficoll-Paque and centrifuged at 900 x g for 30 minutes. The PBMC layer was mixed with RPMI in a new tube and centrifuged an additional three times, at 400 x g for 10 minutes, 8 minutes and 5 minutes respectively.

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2.4 Preparation of cell nuclear fractions

Cells were washed twice with PBS and resuspended in an ice-cold lysis buffer (10 mM Tris, pH 7.4, 1 mM EGTA, 200 mM sucrose) with protease inhibitors (Sigma Aldrich). The lysates were centrifuged for 5 min at 18000 x g at 4˚C and disrupted by sonication on ice 3 x 3 seconds. The protein concentration in the lysates was measured using a Bradford protein assay.

2.5 SDS-PAGE and Western blot

The lysates were treated with Radioimmunoprecipitation assay buffer (RIPA) (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 0.5 mM PMSF) and gel sample buffer containing 12% sodium dodecyl sulfate and 10% β-mercapto-ethanol. Samples were placed in boiling water for 10 minutes and cooled down on ice before loading onto a gel. Typically 15 µg of proteins were loaded onto 12% Tris-Glycine gel and run at 35 mA for about 2 hours Proteins were then transferred to PVDF membranes which were then blocked in 10% non-fat milk for 2 hours at room temperature. Membranes were then incubated in primary mouse anti-human VDR antibody (SantaCruz, clone D6, used at 1:200 dilution) in 5% non-fat milk overnight at 4ºC. Membranes were washed and incubated subsequently in secondary antibody (Cell Signalling, horse anti-mouse IgG HRP linked, used at 1:3,000 dilution) in 5% non-fat milk at room temperature for 2 hours. The protein bands were visualized with chemiluminescence.

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2.6 Determination of cell apoptosis and necrosis using PhiPhiLux and propidium iodide

Cells were stimulated for the indicated periods of time and following the incubation were washed with PBS by centrifugation at 400 *x g* for 5 minutes. The supernatant was discarded and cells were resuspended in 60 µl of PhiPhiLux solution and incubated for 30 minutes at 37°C. PBS was added to each well and the plate centrifuged at 400 *x g* for 5 minutes. Cells were resuspended using flow cytometry buffer (supplied in the PhiPhiLux kit) and 2 minutes before running the samples on the cytometer, propidium iodide was added to a final concentration of 5 µg/ml.

2.7 Determination of cell proliferation using eFluor450 proliferation dye

PBMCs were resuspended in PBS to a concentration of 20 x 10^6 cells/ml and labelled with eFluor450 (20 µM). The cell suspension was incubated at 37°C (protected from light) for 20 minutes. To stop the reaction 10 ml cold complete media was added to the cells and left for 5 minutes. The cells were centrifuged 3 times (400 x g for 5 minutes) in 30 ml of ice-cold complete medium. After the final wash the cells were plated in 96-well plate along with test compounds or vehicle. Cells were incubated for 3, 5 and 7 days in the presence of Protein A from *Staphylococcus aureus* (SAC, 1:10,0000) and Interleukin-2 (IL-2, 20 units/ml). The cells were washed twice with PBS and stained with mouse anti-human CD19 antibody PE conjugated (BD) for 20 minutes at 4ºC. The cells were again washed and resuspended in 1% FBS in PBS buffer, before assay by flow cytometry (with the addition of a known number of counting beads).

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2.8 Statistical analysis

Where applicable multiple comparisons statistical tests were performed in GraphPad Prism 6 software using one-way ANOVA which was followed by the Bonferroni post-test.

3. *Results and discussion*

There are already a few reports describing the impact of vitamin D on B-cells. It is known that normal B-cells express VDR at very low levels though the protein is up-regulated following the stimulation of the cells [15]. Malignant B-cells express VDR and both Hodgkin's and Non-Hodgkin's lymphoma cells have relatively high levels of this protein [16]. It has been shown that 1,25D_3 inhibits proliferation of anti-CD40, anti-IgM, and IL-21 stimulated B-cells [17]. 1,25D_3 has also been shown to up-regulate the production of IL-10 in stimulated B-cells [18]. Of relevance, it has been shown recently that vitamin D deficiency has been associated with significantly poorer outcome in DLBCL patients treated with rituximab [19].

3.1 VDR is expressed by DOHH2 cells and its nuclear translocation is induced after application of VDAs

Expression of VDR in nuclear fractions of DOHH2 cells was assessed after stimulation with 1,25D_3 or various VDAs (all at 10 nM) for 48 hours. HL-60 cells were included as a positive control (Fig. 2). 1,25D_3 and the VDAs all increased the expression of VDR protein. Similarly VDR up-regulation in the nuclear fraction by 1,25D_3 was evident in HL-60 cells (Fig. 2).

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It has to be noted that there is a discrepancy in the literature between binding affinities of the VDAs to VDR and their biological profile. Other analogues of vitamin D have been reported to bind to the receptor with much lower affinities than the active form of vitamin D, yet some of them had more pronounced biological activities (e.g. anti-proliferative properties, lower calcemic effects) than the parent compound [20, 21]. One of the possible explanations for this phenomenon is that upon binding to the VDR downstream signaling actions differ, which manifests differences [22] Alternatively the discrepancy between the binding affinities and the biological activities of the VDAs may be explained by their differential susceptibility to metabolism by CYP24A1. Indeed it may also be relevant that the expression of this enzyme is differentially induced in cell lines by structurally distinct VDAs [23].

3.2 VDAs are cytotoxic to DOHH2 and HL-60 cells

The impact of 1,25D_3 and VDAs upon the viability of the DOHH2 and HL-60 cells was assessed at different time points (24, 48 and 72 hours). 1,25D_3 and VDAs (100 nM) were toxic to cells with cell death primarily associated with necrosis rather than apoptosis. DOHH2 cells were most prone to the VDAs following 24 hours treatment however no clear differences were evident between different analogues (Fig. 3 A). At the two later time points we assume that the compounds have lost their activity (perhaps due to metabolism) and that the cells affected at the early stages of culture have died and hence do not for part of the analyses at 48 and 72h. On the contrary, the viability of HL-60 cells decreased with time and was the lowest after 72 hours (Fig. 3 B). In K422 cells cytotoxicity of vitamin D and some analogues – albeit less

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pronounced that in DOHH-2 cells - has been retained as was the impact of staurosporine, suggesting the greater resistance of the cell line (Fig. 3 C)

3.3 VDAs inhibit the proliferation of stimulated CD19+ B-cells

Next, we examined the effects of 1,25D$_3$ and VDAs on anti-CD19 labelled B-cells from PBMCs obtained from healthy donors. B-cells within the PBMC population were induced to proliferate in response to SAC/IL-2 [24]. The cells were kept in culture for 7 days to assess the impact of VDAs (1, 10 and 100 nM; Fig. 4)

1,25D$_3$ and VDAs decreased the number of proliferated CD19+ after 7 days in a concentration-dependent manner indicating that VDAs inhibit the proliferation on SAC/IL-2 stimulated normal B-cells, which potentially may serve as beneficial in diseases diagnosed with over proliferating B-cells, such as systemic lupus erythematous (SLE) or rheumatoid arthritis (RA) [25, 26].

3.3 VDAs and clomipramine cooperate in their anti-proliferative effects

Many studies have demonstrated that 1,25D$_3$ and some analogues can enhance the anti-proliferative efficacy of various chemotherapeutics in different cancer cells [9, 27]. In addition, with the realisation that a single drug modality – a single 'magic bullet' – is unlikely to offer complete molecular remission, we have also investigated potential synergies of VDAs with other drugs. In the present study we report the VDA combination with the anti-depressant drug clomipramine. Clomipramine has been shown to be cytotoxic and pro-apoptotic against glioma cells [28] and anti-proliferative against malignant, and to a lesser extent, normal B-cells [29, 30]. In previous studies [30, 31] 5 - 10 μM clomipramine was used to demonstrate activity in proliferation assays. The goal of the polytherapy is to obtain similar or higher potential therapeutic outcome with lower adverse effects by combining 2 or more

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active compounds in lower concentrations. Therefore clomipramine was used here at 1 μM. In the present study we applied clomipramine with a range of $1,25D_3$ and VDAs concentrations (1, 10 and 100 nM) to SAC/IL-2 stimulated PBMCs for 7 days and demonstrated an increase in the action of the drug combination in comparison with either drug alone (Fig. 5), however these increases did not reach statistical significance.

4. Conclusions

In summary, this study demonstrates that relatively high concentrations of $1,25D_3$ and vitamin D analogues are cytotoxic to DOHH2 cells (with less pronounced effect in K422 cells) with the extent of killing similar to that observed against HL-60 cells. We have also shown that DOHH2 cells express VDR and that ligand engagement increases expression. From this, we conclude that DOHH2 is a good model to study the effects of $1,25D_3$ and derived compounds in DLBCL. VDAs also inhibited the proliferation of SAC/IL-2 stimulated normal B-cells in a concentration-dependent manner. This effect was enhanced when the analogues were used in combination with the tricyclic anti-depressant clomipramine. Together, this work identifies the potential of using VDAs in a variety of malignant and non-malignant B-cell diseases.

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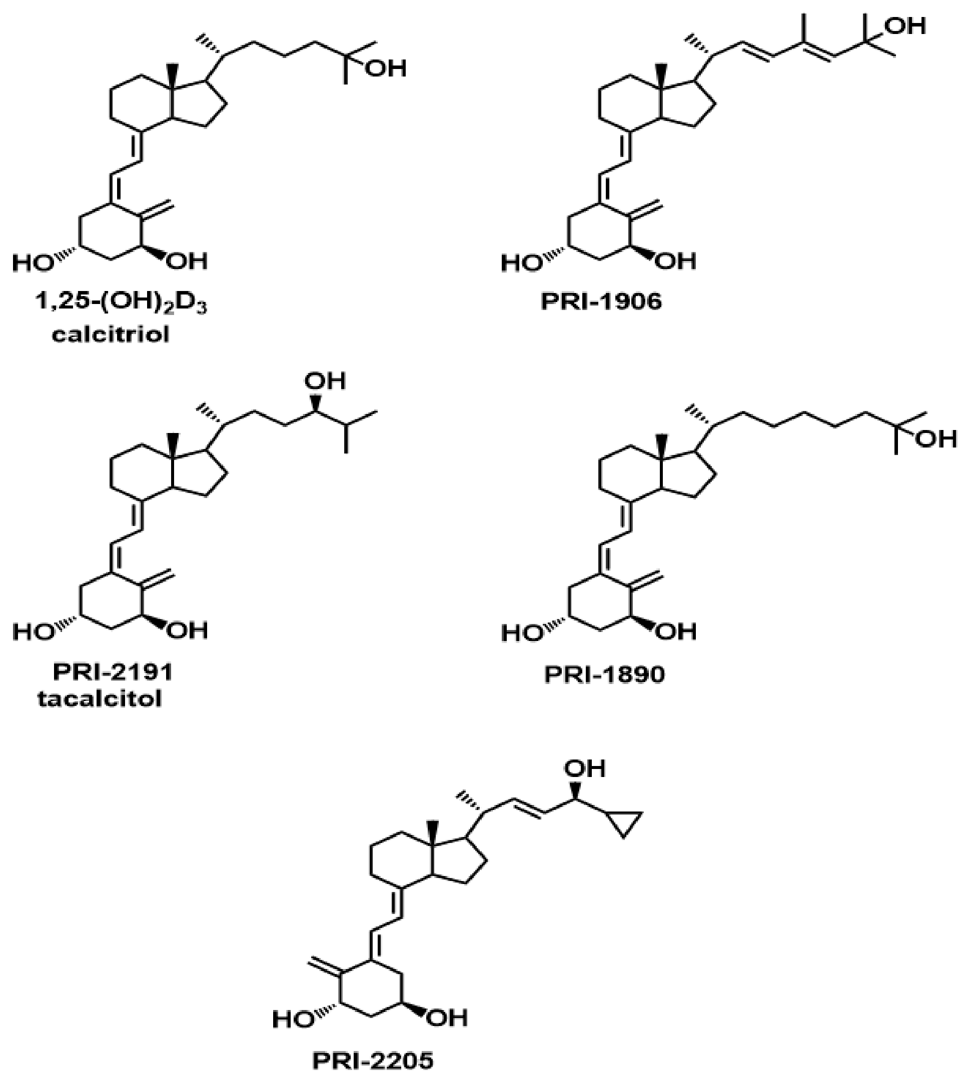

Figure 1. Structures of calcitriol (1,25D$_3$) and vitamin D$_2$ analogues that were used in the present study.

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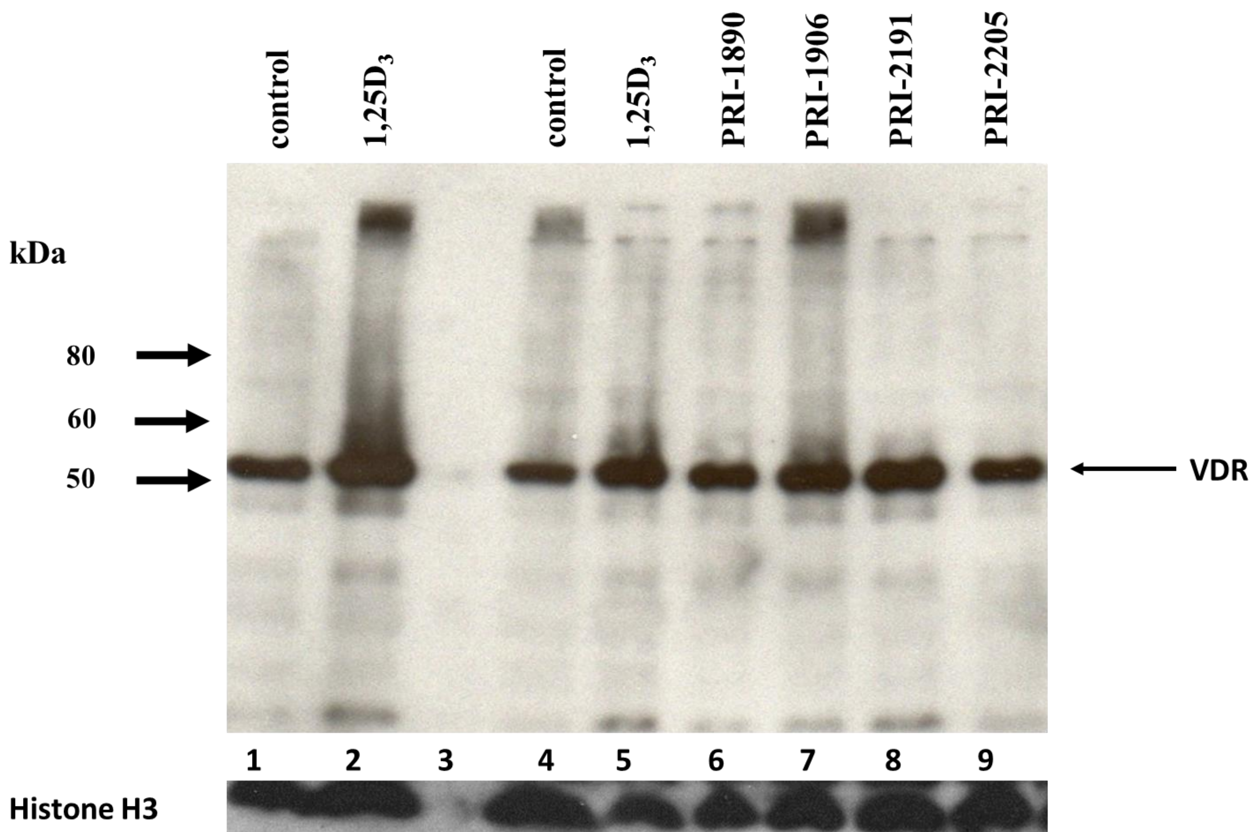

Figure 2. Expression of VDR immunoreactivity in nuclear fractions of HL-60 (lanes 1 and 2) and DOHH2 (lanes 4-9). Cells were treated for 48 hours with 10 nM of each compound or vehicle control. 15 µg of protein were loaded for each well. Histone H3 immunoreactivity was used as a loading control. A representative example of two independent experiments with similar results is shown.

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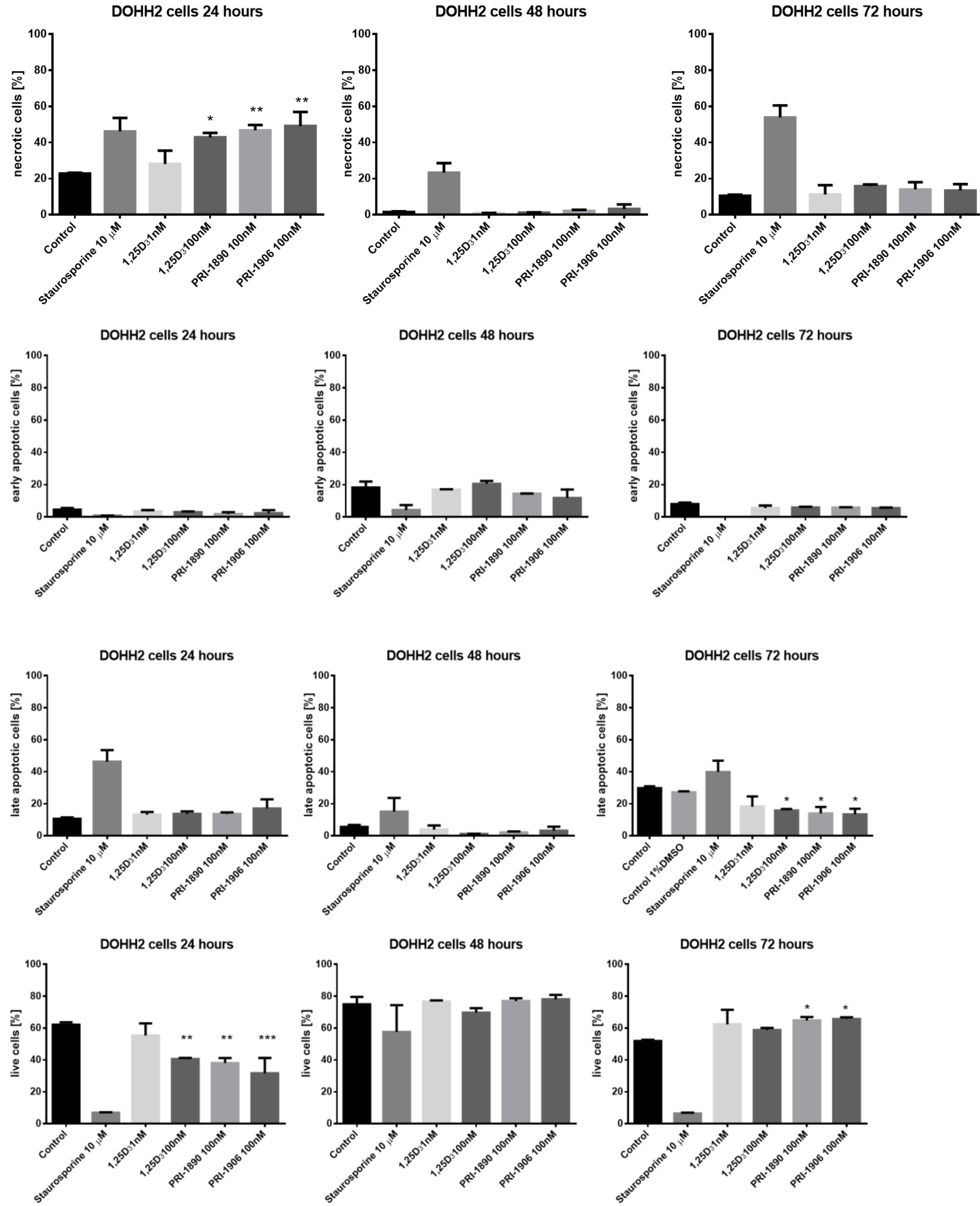

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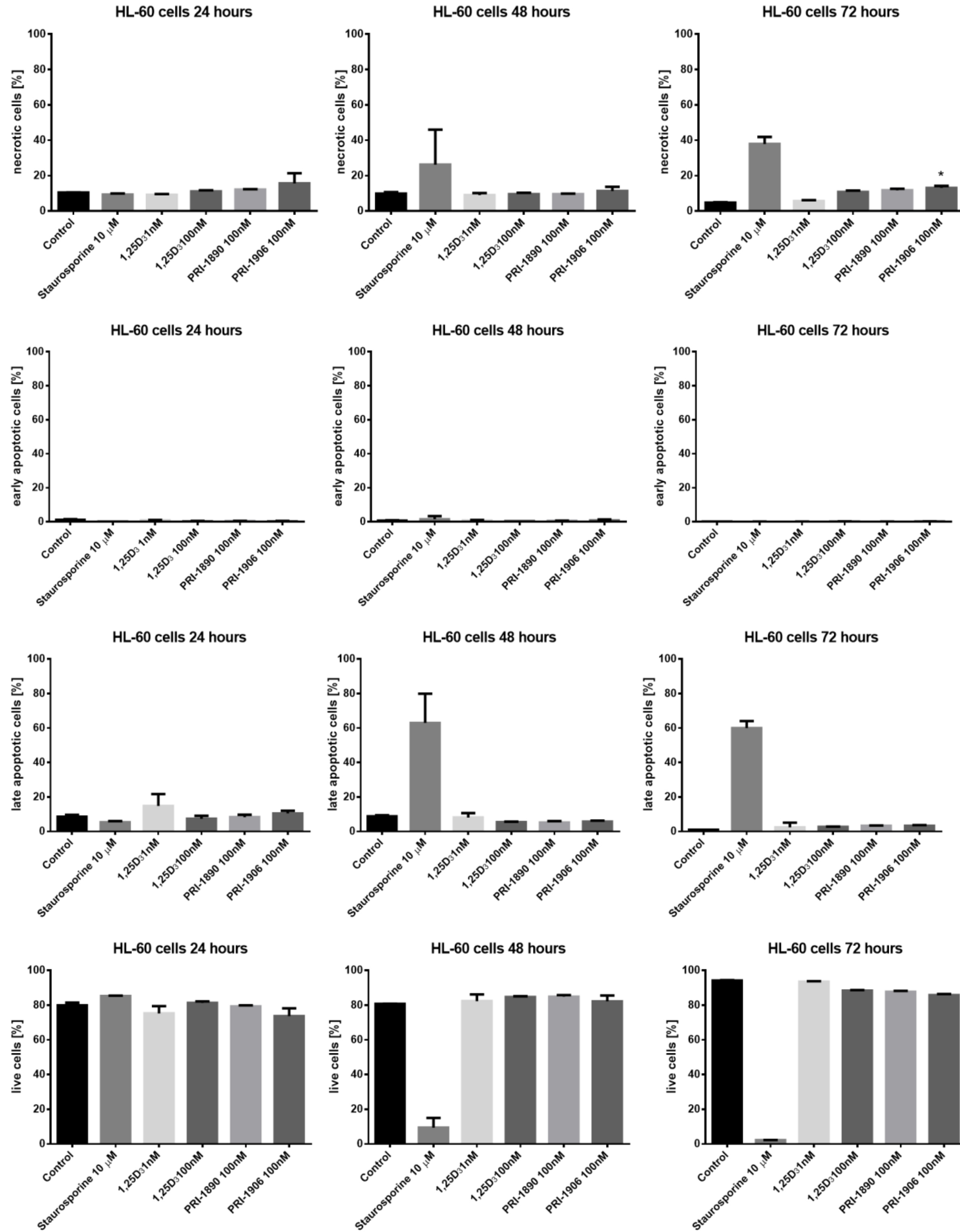

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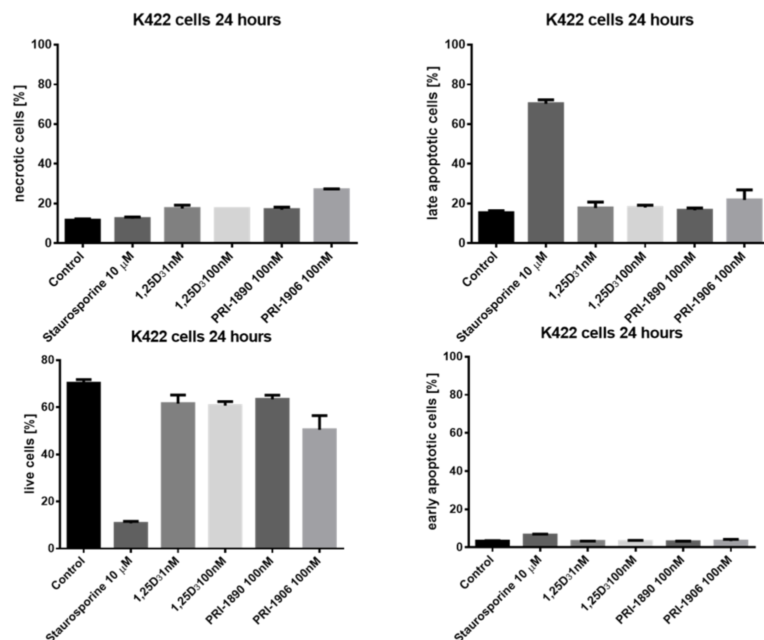

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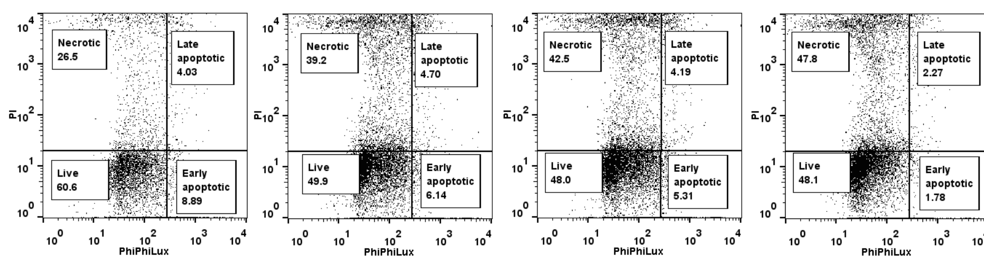

*Figure 3. Impact of 1,25D$_3$ and some VDAs upon viability of (A) DOHH2, (B) HL-60 cells and (C) K422 cells following different treatment for 3 different time periods assayed by PhiPhiLux assay. PRI-1906 markedly increased the number of necrotic HL-60 cells after 72 hours. Representative flow cytometry plots (D) for DOHH2 cells (from left to right: control, 1,25D$_3$, PRI-1890 and PRI-1906 at 100 nM). Data represent mean ± SD, n=3. *** ($P < 0.01$), ** ($P<0.05$), * ($P<0.1$) compared with Control.*

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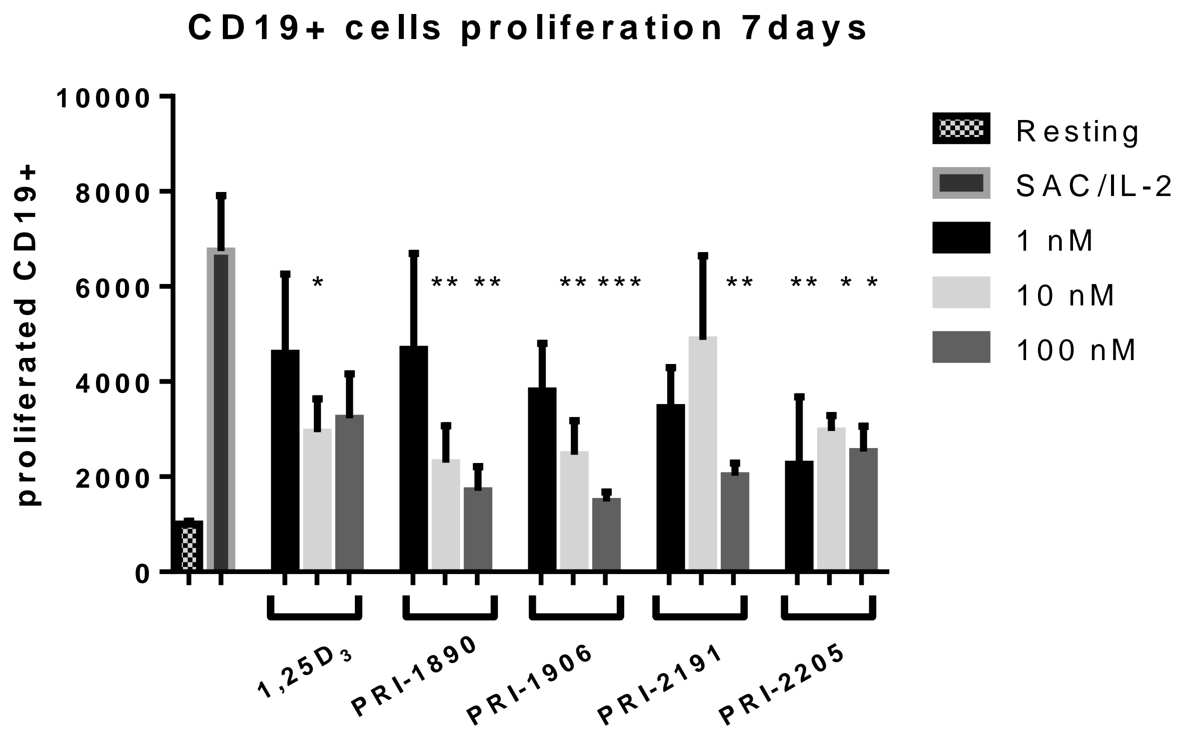

*Figure 4. VDAs have anti-proliferative effects on SAC/IL-2 stimulated CD19+ B- cell (7 days). Data represent mean ± SD from 3 independent experiments.*** (P < 0.01), ** (P<0.05), * (P<0.1) compared with SAC/IL-2 stimulated cells.*

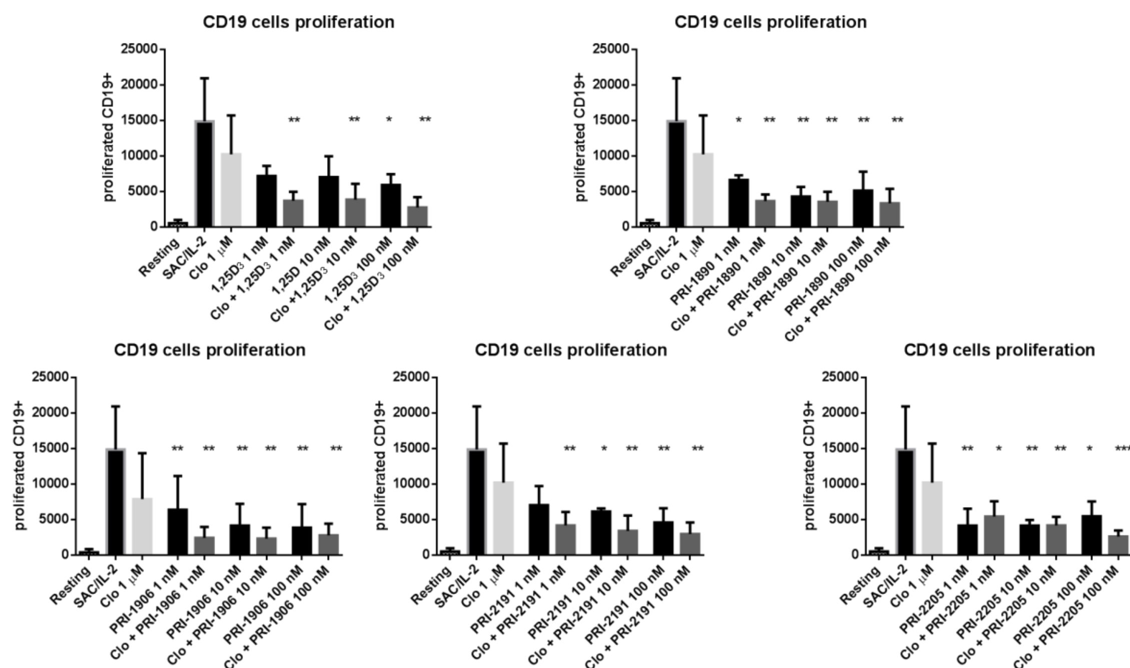

*Figure 5. Additive anti-proliferative effect of the combination of 1,25D$_3$ and VDAs (indicated concentrations) with clomipramine (Clo; 1 μM) on SAC/IL-2 stimulated CD19+ B-cells. Cells were stimulated for 7 days and labelled with CD19 antibody. Data represent mean ± SD from 3 independent experiments.*** ($P < 0.01$), ** ($P<0.05$), * ($P<0.1$) compared with SAC/IL-2 stimulated cells. No statistically significant differences were found when the combinations of VDAs wth Clo were compared to VDAs used alone.*